

# Anaerobic storage of red blood cells

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## Introduction

In 2007, approximately 56 million units of blood were collected in developed countries. In these countries (~25% of the world's population), the common risks associated with blood transfusions are minimised by careful screening of donors, extensive testing of units for infectious agents and routine cross-matching. Well-developed logistics enable the level of wastage due to outdating of stored units to be kept low.

Recent clinical studies demonstrating a negative correlation between RBC transfusions and clinical outcomes have led to a significant attention being focused on the quality and efficacy of stored blood used in transfusion therapy<sup>1-16</sup>.

In RBC separated from whole blood and stored in a refrigerator away from the turbulent environment of circulation, the normal senescence processes are suspended. When stored RBC are returned to the circulation, a normal life-span is observed but only for cells not removed by the reticuloendothelial system of the recipient shortly after reinfusion<sup>17</sup>. After 6 weeks of storage, up to 25% of stored cells are removed within 24 hours of infusion due to the damage inflicted on the RBC during their collection, component preparation and refrigerated storage. This damage ("storage lesion") manifests as changes in biochemical and physical parameters of RBC as well as in-storage haemolysis and reduced post-transfusion 24-hour recovery *in vivo*.

The clearance of damaged RBC reduces the therapeutic efficacy of the unit, stresses the reticuloendothelial system of the recipient and adds an excess iron burden to chronically transfused patients. Moreover, transfusion of damaged RBC is suspected as a possible cause of clinically observed complications of transfusion therapy. Current controversy surrounding the possible causal

relationship between the "age" of units and negative outcomes in patients receiving transfusions<sup>13,15,16,18-41</sup> stems from the fact the most of the storage-related damages increase with storage duration.

In the past, various strategies were suggested and tested to reduce the rate of development of storage lesions. These strategies can be classified into four categories: (i) manipulation of pH; (ii) supplementation of metabolic precursors; (iii) manipulation of osmotic balance and increase of the volume of the suspending medium; and (iv) reduction of oxidative stress by adding protective molecules. Several studies employing a combination of these strategies have reported maintenance of viability allowing an extension of storage beyond the current limit of 6 weeks<sup>42-47</sup>.

Our approach to improving the quality and efficacy of stored RBC has been focused on reducing oxidative damage by removing oxygen at the beginning of storage and maintaining the anaerobic state throughout the storage period<sup>48-52</sup>.

In this article, the mechanisms of oxidative damage, its involvement in the development of storage lesion, as well as various attempts to reduce such damage are summarised. Emphasis is placed on reviewing results of a series of preliminary clinical studies conducted by our group to investigate the effect of anaerobic storage to reduce such damage by storing RBC under oxygen-depleted conditions.

## Red blood cell storage lesions and their effect on erythrocyte function

### Physiological function of red blood cells

One of the primary functions of RBC is to transport oxygen (O<sub>2</sub>) from the lungs to metabolising tissues: O<sub>2</sub> diffuses from the alveoli into the pulmonary capillaries, binds to haemoglobin (Hb) in RBC and dissolves in plasma, and is then transported with the

flow of blood to the microvascular networks where it is released from Hb, diffuses across the walls of capillaries, pre-capillary arterioles and post-capillary venules to the cells of tissues<sup>53-57</sup>. To maintain adequate perfusion of microvascular networks, RBC must be able to continually deform at physiologically high haematocrits, under a wide range of flow conditions, in vessels with diameters ranging from 3-8  $\mu\text{m}$  (capillaries) to 50-100  $\mu\text{m}$  (arterioles and venules). Maintaining an appropriate level of "deformability" is, therefore, crucial for RBC physiological function<sup>58,59</sup>.

In addition to transporting  $\text{O}_2$ , RBC can sense the state of local tissue oxygenation and adjust the rate of  $\text{O}_2$  delivery by regulating the local blood flow in microvascular networks<sup>60-62</sup> via three possible nitric oxide (NO)-mediated mechanisms: (i) release of adenosine triphosphate (ATP) to stimulate production of NO by the endothelial cells lining the walls of the vessels<sup>63-68</sup>; (ii) release of NO from S-nitroso- (SNO) Hb upon deoxygenation of Hb<sup>69-72</sup>; and (iii) reduction of nitrite ( $\text{NO}_2^-$ ) present in the blood stream to NO by deoxyhaemoglobin<sup>73-75</sup>. Vessels dilate in response to the release of NO, thus changing their fluidic resistance and consequently the rate of blood flow.

### The effect of storage on red blood cell functions

In 4°C liquid storage, the biochemical and mechanical properties of RBC deteriorate progressively. These "storage lesions" affect all of the key RBC functions<sup>15,36,71,76</sup>.

### Loss of viability

RBC stored for a period of time at 4°C lose viability. Some may undergo spontaneous haemolysis while in storage; others lose the ability to survive in the recipient's circulation following transfusion. The survival of transfused RBC is usually measured by infusing subjects with a sample of stored RBC tagged with a radioactive label ( $^{51}\text{Cr}$ ), and then sampling the blood of the recipient periodically to determine the kinetics of RBC clearance<sup>51,77-79</sup>. A sizable portion of transfused RBC is cleared from the circulation of the recipient in the first 24 hours after transfusion, and the number of these non-viable RBC increases with duration of storage. Transfused RBC that still remain in circulation after this initial period of rapid clearance will have relatively normal life-spans. Currently, the

US Food and Drug Administration requires that stored RBC experience less than 1% in-storage haemolysis and that at least 75% of stored RBC survive in the recipient 24 hours after transfusion<sup>80,81</sup>.

### Biochemical changes

#### Adenosine triphosphate

When RBC are stored in additive solutions, the concentration of ATP remains level or even increases early in storage, peaks at about 2 weeks, but then gradually declines to below 50% by week 6 of storage<sup>82-84</sup>. Anaerobic oxidation of glucose (i.e., glycolysis) is the only source of energy for RBC. The initial steps of this process require ATP; it cannot continue when ATP becomes depleted. The fall of ATP levels and of the total adenylate content (ATP+ADP+AMP) is associated with poor *in vivo* survival<sup>85</sup>. The loss of ATP may also diminish the ability of transfused RBC to effect NO-mediated arteriole vasodilatation in response to hypoxia<sup>64,86,87</sup>.

#### 2,3-diphosphoglycerate

The 2,3-diphosphoglycerate (2,3-DPG) levels decline rapidly over the first week of storage, falling to undetectable levels by the end of week 2<sup>15,71,83,84</sup>. Because of the loss of 2,3-DPG, stored RBC release  $\text{O}_2$  to the tissues less readily than normal cells. After transfusion, however, 2,3-DPG is rapidly re-synthesised to 50% of the normal level in as little as 7 hours, and to 95% of the normal level in 2-3 days<sup>36,88-91</sup>. This restoration of 2,3-DPG returns the Hb- $\text{O}_2$  dissociation curve of stored RBC back to its normal position, and thus normalises the amount of  $\text{O}_2$  functionally available to tissues.

#### S-nitroso haemoglobin

SNO-Hb is lost precipitously in the first few hours of storage, which may affect the ability of RBC to regulate the local blood flow in response to low  $\text{pO}_2$ <sup>71,92</sup>. SNO-Hb, however, recovers within several hours in the body<sup>15</sup>. Also, it may be not essential for the RBC-dependent hypoxic vasodilatation<sup>72</sup>.

### Changes in mechanical properties

During storage, some RBC change shape from deformable biconcave disks to echinocytes to spherocytes<sup>36,93</sup>. The number of the irreversibly deformed spherocytes in the population of stored RBC

gradually increases throughout the duration of storage<sup>93</sup>. The significantly reduced surface area-to-volume ratio of spherocytes limits their ability to pass through the smallest capillaries<sup>94</sup>; spherocytes are quickly culled from circulation by the body of the recipient<sup>84,95</sup>.

RBC gradually lose their "deformability" during storage – specifically, the ability to undergo folding deformations (the kind 8- $\mu$ m RBC need to be able to do to traverse 3-7  $\mu$ m capillaries)<sup>83,93,96-100</sup>, and shear deformations (the kind RBC will experience in larger vessels, arterioles and venules)<sup>101-106</sup>. Because of this storage-induced degradation of their mechanical properties, stored RBC may be unable to maintain adequate blood flow in capillaries and deliver oxygen as effectively as their fresh counterparts. If transfused, stored RBC may be unable to improve perfusion of the microvascular networks and oxygenation of tissues immediately after transfusion, and this will reduce the clinical efficacy of RBC transfusion<sup>10,107,108</sup>.

### **Mechanisms of oxidative damage incurred by stored red blood cells**

Oxidative damage – lipid and protein oxidation / peroxidation and cross-linking caused by reactive oxygen species, such as hydroxyl, peroxy and alkoxy radicals – is one of the major factors contributing to the development of storage lesions. RBC contain a highly reactive mixture of iron (in Hb) and oxygen (dissolved in the cytosol and bound to Hb) at very high concentrations. Iron atoms in Hb must be maintained in a ferrous state in order to reversibly bind O<sub>2</sub>. *In vivo*, ferrous iron in Hb is protected from oxidation within RBC, but when the cells are removed from the body, and stored under refrigerated conditions, the mechanisms protecting the RBC lose their efficiency and Hb becomes vulnerable to oxidation.

#### ***Haemoglobin oxidation and the oxidative damage pathway***

Haemoglobin and the products of its denaturation play a central role in the development of oxidative damage to stored RBC by serving as catalysts of this process. Although the importance of denatured Hb in the context of oxidative damage during refrigerated storage was raised back in 1989 by Wolfe<sup>109</sup>, relatively few studies that address this issue directly have been

reported since. The involvement of Hb in the development of storage lesions has been reviewed recently<sup>110</sup>.

Haemoglobin contains four ferrous ions, one in each of its four subunits coordinated in the porphyrin ring. To fulfil its main physiological function, each deoxyHb molecule binds four molecules of O<sub>2</sub> reversibly, without exchanging electrons (Figure 1A). RBC maintain a highly reducing environment in their cytosol to preserve iron in its ferrous form and use enzymes efficiently to reverse Hb oxidation.

A small fraction of oxyHb spontaneously auto-oxidizes to form ferric methaemoglobin (metHb) and a superoxide anion. In circulation, ferric metHb is reduced back to ferrous Hb by NADH-linked cytochrome b<sub>5</sub> reductase. In refrigerated storage, however, this reaction is retarded while the formation of metHb is enhanced for stored RBC with partially oxygenated Hb (Figure 1B). Once formed, metHb is inherently unstable; it denatures readily first to reversible hemichromes, then to irreversible hemichromes, and finally to globin and free haem (haemin; Figure 1C). The stability of metHb may be further compromised at storage temperatures similar to a lower thermodynamic stability of met-myoglobin at 4°C (cold denaturation)<sup>111</sup>.

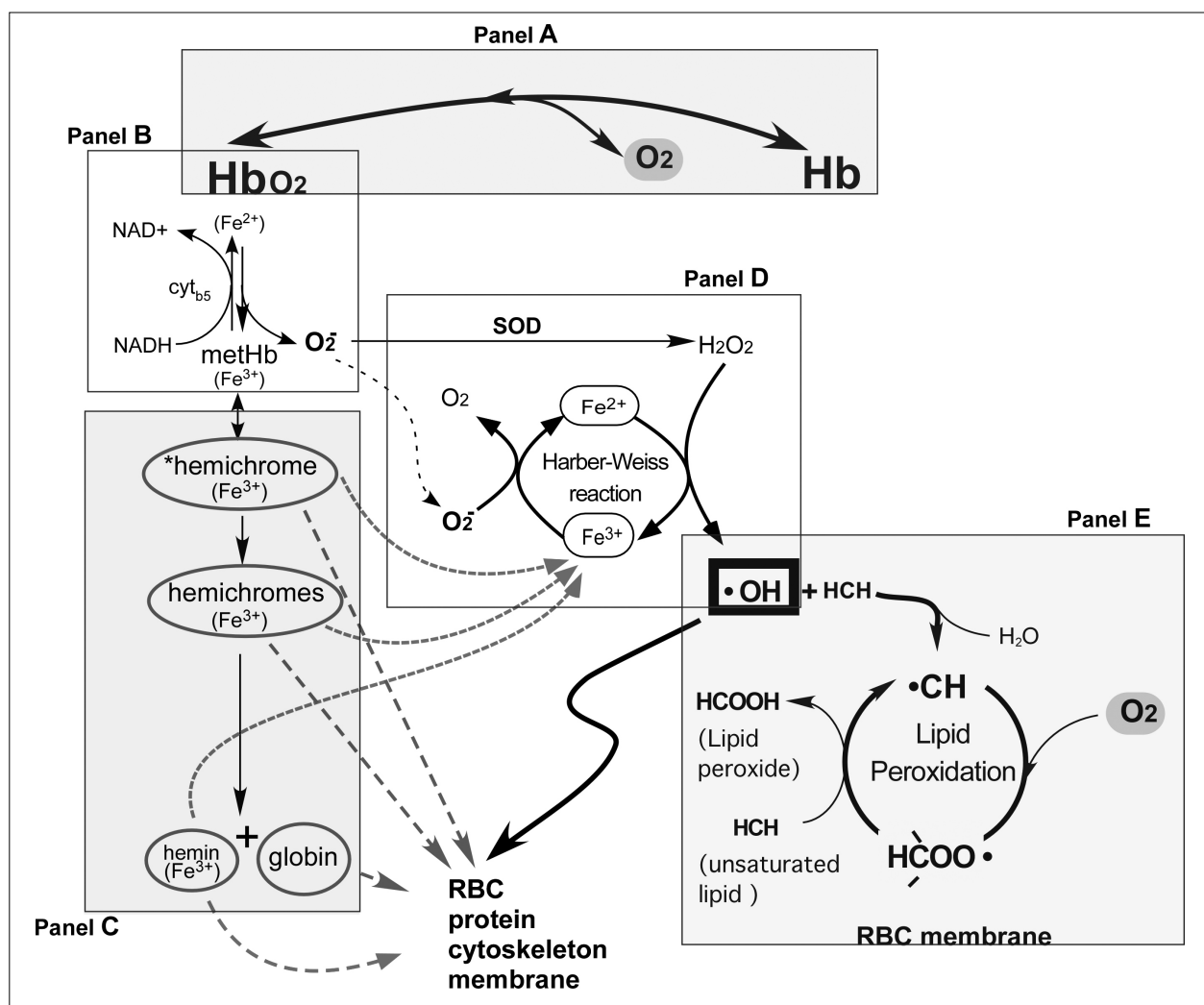
Ferric iron in hemichromes, free haem and molecular iron released from haem can all function as a Fenton reagent in the Harber-Weiss cycle fed by H<sub>2</sub>O<sub>2</sub> to generate highly reactive hydroxyl radicals (Figure 1D). Hydroxyl radicals ( $\bullet$ OH) attack proteins and (in the presence of oxygen) initiate a lipid peroxidation cycle in RBC membrane (Figure 1E). Polyunsaturated fatty acids in lipids are attacked by  $\bullet$ OH to form lipid radicals, which then form lipid hydroperoxyl radicals with oxygen, which in turn attack polyunsaturated lipid to complete the cycle. This cycle continues in presence of oxygen until two radicals react to terminate the reaction, resulting in cross-linked lipids<sup>112</sup>.

#### ***Haemoglobin auto-oxidation under conditions of partial oxygen depletion***

H<sub>2</sub>O<sub>2</sub> is a substrate for the Harber-Weiss reaction that produces hydroxyl radicals and itself is a product of superoxide dismutase reacting on superoxide anion, a byproduct of Hb auto-oxidation (Figure 1B and D). Because metHb forms when oxyHb auto-oxidizes, the

concentration of dissolved  $O_2$  is a critical factor in determining the rate of metHb formation. Balagopalakrishna *et al.* reported that the rate of metHb formation and superoxide production reached a maximum when Hb was only partially occupied by  $O_2$  in a hypoxic state ( $SO_2 \sim 60\%$ ) rather than fully oxygenated at a high  $pO_2$ <sup>113</sup>. In this context, the current

practice of collecting and processing venous blood, and placing it in storage at a starting  $SO_2$  of nearly 60% and then allowing the blood to oxygenate further to nearly 100% during the 6 weeks of storage as oxygen slowly diffuses through the plastic film of the storage bag, likely exacerbates the oxidative damage experienced by stored RBC.



**Figure 1 -** Haemoglobin and pathways of oxidative damage in RBC.

**Panel A:** Normal function of Hb-reversible binding of  $O_2$  to reduced (ferrous) haems in Hb. **Panel B:** Auto-oxidation of oxyHb to methaemoglobin (metHb; ferric) with production of superoxide anion. In a steady state, 1-2% of Hb exists as metHb in the circulation; metHb is readily reduced back to ferrous Hb by NADH-linked cytochrome  $b_5$  metHb reductase. **Panel C:** Denaturation of metHb. MetHb denatures first to 'reversible hemichromes', in which conformational distortions are minor and can still be reversed. Reversible hemichromes further denature to 'irreversible hemichromes', which subsequently dissociate to globins and the haem moiety. **Panel D:** The Harber-Weiss reaction produces hydroxyl radicals,  $\cdot OH$ . Superoxide anions generated in the production of metHb are converted into  $H_2O_2$  by superoxide dismutase. Hydroxyl radicals are produced with  $H_2O_2$  and ferrous iron from denatured metHb products functioning as Fenton reagents. Ferric iron is reduced by superoxide anions. Hydroxyl radicals oxidize and cross-link RBC proteins in their vicinity. **Panel E:** Lipid peroxidation cycle. Hydroxyl radicals in the membrane attack unsaturated lipids to form lipid radicals, then combine with molecular oxygen to form lipid peroxyl radicals, which in turn attack unsaturated lipid to complete the cycle.

### ***The effect of oxidative damage on stored red blood cells***

Obtaining unequivocal direct evidence of oxidative damage to RBC during refrigerated storage has been difficult prior to the application of highly sensitive and specific proteomic techniques<sup>114,115</sup>. For example, a simple measurement of metHb levels may not accurately reflect the rate of metHb formation or the extent of oxidative reactions during storage due to the transient nature of unstable metHb. A small fraction of RBC that were nearly senescent at the time of blood collection and damaged RBC may cause high readings of free Hb, haem and other by-products of oxidation. Thus, most available evidence regarding the mechanisms of Hb-mediated oxidative damage and their impact on the physiological function of RBC were obtained from *in vitro* experiments exposing RBC to oxidative stress<sup>104,116-121</sup> or from studying RBC from patients with various haemoglobinopathies (such as sickle cell disease or thalassaemia)<sup>122-125</sup> and other conditions (such as glucose-6-phosphate dehydrogenase deficiency or myelodysplastic syndrome)<sup>126,127</sup>.

During conventional (aerobic) storage, a progressive increase in overall protein oxidation has been observed<sup>128-130</sup>. D'Amici *et al.* demonstrated a significant *reduction* in the rate of increase in the number of spots on a two-dimensional gel of RBC proteins (an indicator of protein modification and fragmentation) in the first week of storage for anaerobically stored RBC relative to those stored aerobically (conventionally), suggesting a reduced rate of RBC protein oxidation under anaerobic conditions. By employing standard proteomic techniques, they also identified proteins that were modified early and in an O<sub>2</sub>-dependent manner, including band 4.2 (prevalent), band 4.1, band 3 and spectrin (minor)<sup>114</sup>. Messian *et al.* observed a loss of oxygen-dependent metabolic modulation in RBC stored aerobically<sup>131</sup>. In addition to these physical and chemical perturbations, a decrease in glutathione (GSH) levels and in activity of GSH-dependent glutathione peroxidase were observed during storage<sup>132</sup>.

After Hb auto-oxidizes to metHb, it denatures to hemichromes, which then precipitate onto the lipids and the cytoskeleton of RBC membrane. Subsequent clustering of band 3 caused by this precipitation allows the immune system of the recipient to recognise and

cull the transfused RBC from the circulation<sup>109</sup>. Precipitated hemichromes also disrupt the interactions between other proteins of the cytoskeleton (spectrin, actin and band 4.1)<sup>109,133</sup>. Hemichromes undergo further denaturation into haemin, globin and free molecular iron, all of which readily partition into lipid bilayers. Free haem is a potent haemolytic agent; it causes RBC haemolysis by collapsing the cationic gradient<sup>134</sup>. Haemin and free molecular iron can also function as Fenton reagents to generate hydroxyl radicals, which in turn initiate lipid and protein peroxidation reactions. Peroxidation of the lipids and proteins of the RBC membrane results in an increased susceptibility to cation leaks, elevated phosphatidyl serine exposure, vesiculation and haemolysis. All of these changes prime RBC for suicidal cell death (eryptosis) in the circulation<sup>135</sup>. Excessive vesiculation due to oxidative damage leads to a disproportionately higher loss of membrane area than volume; as the surface area-to-volume ratio decreases, stored RBC change shape, ultimately approaching a sphere. This change in the surface area-to-volume ratio and the cross-linking of membrane lipid and proteins of the cytoskeleton caused by the hydroxyl radicals contribute to the significant reduction of deformability of stored RBC<sup>93,96,109,118,120,124,125</sup>.

### **Prevention of oxidative damage during red blood cell storage**

#### **Previous attempts to reduce oxidative damage**

There are two possible approaches to reducing the rate of oxidative damage during RBC storage: adding anti-oxidants to the storage solution, or removing oxidants from RBC suspension.

Reducing oxidative damage by addition of anti-oxidants or precursor molecules to augment the RBC's own anti-oxidative mechanisms is an attractive approach that has shown its effectiveness in improving RBC biochemical parameters measured *in vitro*<sup>116,136,137</sup>. For example, Dumaswala *et al.* proposed supplementing additive storage solution with GSH precursor amino acids (glutamine, glycine and N-acetyl-L-cysteine) to prevent the gradual decline in GSH levels during storage, and to maintain a reducing environment in RBC cytosol. Although an enhancement in GSH levels and various other biochemical parameters was observed, the actual impact of these changes on *in vivo* recoveries is not

yet known<sup>138</sup>. Any modification of currently used storage solutions (especially by adding *new* ingredients), however, will require exhaustive studies of safety before gaining an approval for routine use from regulatory authorities (such as the US Food and Drug Administration).

An alternative approach to reducing oxidative damage is to remove O<sub>2</sub> from RBC suspensions in the beginning and throughout storage – thus shutting down the haemoglobin denaturation pathway and arresting all oxidative reactions fuelled by O<sub>2</sub> (Figure 1). This approach is feasible due to a unique feature of RBC: unlike normal eukaryotic cells, RBC rely solely on anaerobic glycolysis (rather than oxidative phosphorylation carried out by mitochondria) for their energy metabolism.

Högman's group reported on the *in vitro* characterisation of RBC stored in a canister filled with N<sub>2</sub> to prevent oxygenation of venous blood during 6 weeks of storage<sup>139</sup>. In that study, RBC were prepared for storage conventionally (i.e., *not* de-oxygenated). By preventing oxygenation of Hb through the wall of the polyvinyl chloride blood bag (which happens slowly during conventional storage), and allowing gradual equilibration of O<sub>2</sub> and N<sub>2</sub> in the canister, they observed a decrease of SO<sub>2</sub> from ~60% to 32% over the 6-week period, compared to an increase in SO<sub>2</sub> to nearly 100% for RBC stored conventionally<sup>139</sup>. These authors reported a higher ATP level and an increased RBC deformability, although no *in vivo* data were collected. However, since the rate of Hb auto-oxidation reaches a maximum at an SO<sub>2</sub> of around 60%<sup>113</sup>, it is doubtful that the procedure used by Högman *et al.* was an optimal approach to reducing oxidative damage.

### Red blood cell storage under strictly anaerobic conditions

Apart from our own work<sup>48-51</sup>, there has been neither systematic nor *in vivo* studies reported in the literature on the storage of RBC in which O<sub>2</sub> is stripped from Hb at the *beginning* of storage and maintained at that level (e.g., in an anaerobic canister) throughout the whole duration of storage.

In our efforts to reduce oxidative damage during RBC storage, we attempted to reduce SO<sub>2</sub> at the beginning of storage to a level as low as practical, and then maintained (and further reduced) SO<sub>2</sub> during

the entire storage duration. This was accomplished by first equilibrating the RBC suspension with Ar gas repeatedly over a period of 1 hour prior to refrigerated storage (SO<sub>2</sub> was reduced to less than 3.6%), then storing RBC in a standard blood bag inside an anaerobic canister filled with Ar and H<sub>2</sub> (9:1) with a palladium catalyst (to further deplete O<sub>2</sub> during the 6 to 9 weeks of storage)<sup>51</sup>.

### Impact of oxygen removal on the development of oxidative damage

Reducing SO<sub>2</sub> to a very low level early in storage may alleviate the extent of oxidative damage being accumulated by RBC via the following possible mechanisms:

- i) reducing the concentration of oxyHb and thus reducing the concentration of metHb produced by auto-oxidation of oxyHb (Figure 1, Panel A-B);
- ii) preventing disruption of the membrane and cytoskeleton caused by hemichromes, haemin and globin (the products of metHb denaturation) and thus reducing haemolysis and eryptosis (Panel C);
- iii) preventing production of free haem and iron (Fenton reagents in the Harber-Weiss reaction for hydroxyl radical production);
- iv) reducing production of superoxide anion (superoxide anion feeds the Harber-Weiss reaction in two ways: as a substrate to reduce ferric iron and as a precursor to H<sub>2</sub>O<sub>2</sub>, Panel D);
- v) reducing production of hydroxyl radicals and subsequent cross-linking of cytoskeletal proteins to prevent reduction in deformability (Panel D);
- vi) curtailing lipid peroxidation reactions (at SO<sub>2</sub>=4%, free oxygen concentration is reduced to less than 1% of air-saturated blood at 4°C, Panel E) to reduce haemolysis and eryptosis.

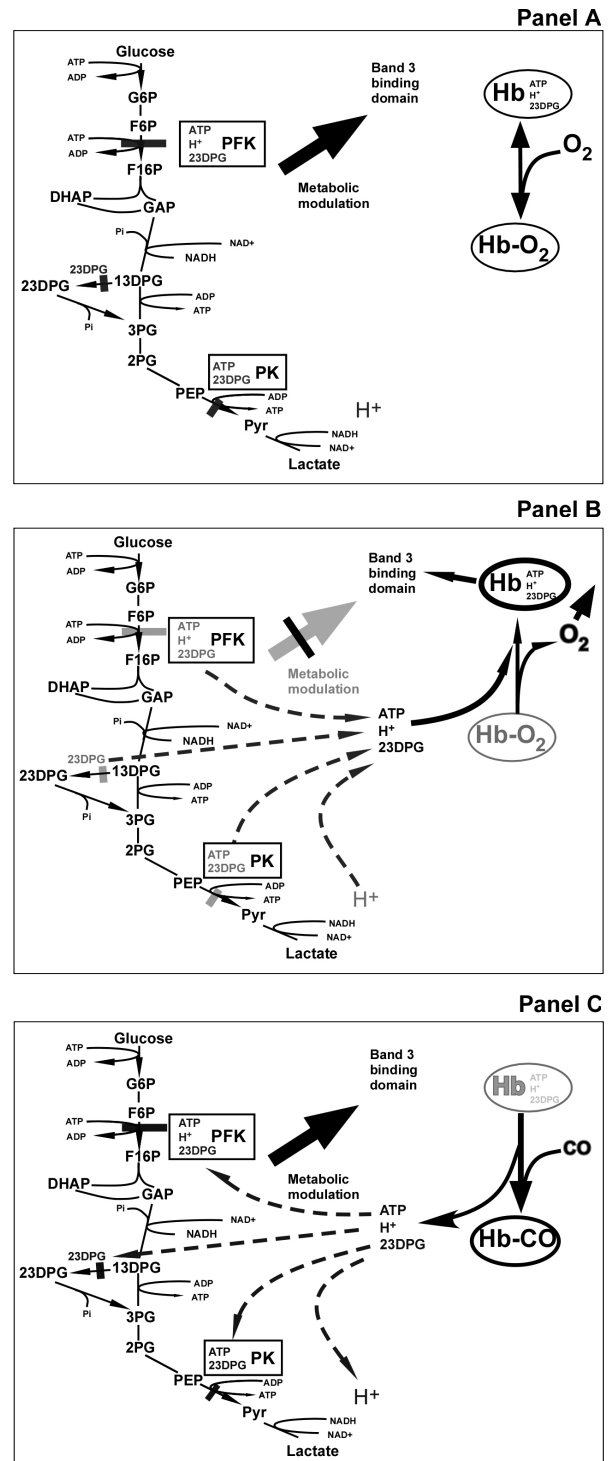
### Metabolic consequences of oxygen removal

An overwhelming fraction of protein contained in the cytosol of RBC is haemoglobin. Consequently, the biochemistry of Hb determines the rates of oxidative reactions and influences the energy metabolism of RBC, and thus Hb plays a primary role in the development of metabolic storage lesions.

Hb is a tetrameric protein that may exist in two conformations with different affinities for O<sub>2</sub> – the high affinity R-state (oxygenated) and the low affinity T-state (deoxygenated). In addition to O<sub>2</sub>, Hb binds other ligands – *heterotropic effectors* – with affinities that

depend on its conformation. Hb exerts control of metabolic reactions by modulating the concentration of free ligands through its own conformational transition.  $H^+$ , 2,3-DPG and (to a lesser extent) ATP are the heterotropic effectors of Hb, which directly affect the production of ATP by the glycolytic pathway and of 2,3-DPG by the Rapoport-Luebering shunt. As illustrated in Figure 2A, ATP and 2,3-DPG function as end-product inhibitors of phosphofructokinase (PFK) and pyruvate kinase (PK). PFK is also inhibited by low pH resulting from lactate production. In addition, Hb modulates the activity of glycolytic enzymes, namely PFK, glyceraldehyde-3-phosphate dehydrogenase, aldolase and lactate dehydrogenase by oxygen-dependent metabolic modulation<sup>140</sup>. When these enzymes bind to the cytoplasmic binding domain of band 3, their activities are inhibited<sup>141</sup>. DeoxyHb (Hb in T-state) competitively binds the same binding domain of band 3, thus releasing PFK and other enzymes, disinhibiting their activity and increasing the glycolytic flux.

When  $O_2$  dissociates from oxyHb, the following events take place: (i) Hb conformation is forced from the R- to T-state; (ii) Hb binds 2,3-DPG at a near 1:1 stoichiometric ratio because the affinity of Hb for 2,3-DPG is higher in the T-state; (iii) deoxyHb binds  $H^+$  (Bohr proton); and (iv) deoxyHb preferentially binds to the cytoplasmic binding domain of band 3, thereby releasing PFK and other glycolytic enzymes (Figure 2B). The increased affinity of deoxyHb to 2,3-DPG and ATP reduces their cytosolic concentrations thus removing inhibition from PFK and PK; uptake of Bohr proton increases cytosolic pH, up-regulating PFK even further. The binding of deoxyHb to the binding domain of band 3 and the consequent displacement of PFK and other glycolytic enzymes also up-regulates glycolytic activity<sup>131,140</sup>. These changes result in an increase in glycolytic flux and activity of 1,3-DPG mutase as indicated by the elevated levels of lactate and ATP (Table I) as well as 2,3-DPG levels in the first two weeks of storage<sup>50</sup>.



**Figure 2** -Oxygenation state of haemoglobin and RBC energy metabolism.

**Panel A:** Hb in oxygenated state. Bars at PFK, PK and 1,3-DPG mutase steps indicate end product inhibitions by ATP, 2,3-DPG and low pH. The large arrow indicates binding of PFK and other glycolytic enzymes to the cytosolic binding domain of band 3 (thus reducing their enzymatic activity). **Panel B:** RBC in anaerobic conditions. Hb in T-state binds 2,3-DPG,  $H^+$  and ATP (to a limited extent) thereby releasing the inhibitory steps (grey bars). DeoxyHb has higher affinity toward the cytoplasmic binding domain of band 3; upon binding to band 3, deoxyHb displaces and thus activates PFK and other metabolic enzymes. **Panel C:** When CO is used in place of Ar to deplete  $O_2$  in gas exchange, CO binds (nearly) irreversibly to Hb, fixing it in R-state and releasing Hb from the binding domain of band 3. This enables PFK and other enzymes to bind to band 3, which effectively removes them from glycolytic reactions. Due to a lower affinity of R-state, Hb releases ATP,  $H^+$  and 2,3-DPG, elevating their free cytosolic concentrations, and inhibiting key enzymes resulting in reduction of glycolytic flux.

**Table I** - Clinical studies of anaerobically stored RBC

Additive solution	Weeks	n storage	SLR (%)	DLR (%)	Glucose (mg/dL)	Lactate (mmol/L)	Morphology (%)	ATP (mmol/gHb)	Hemolysis (%)	DPG (umol/gHb)	Life span (days) <sup>a</sup>
<b>Study I OFAS1</b>											
	8	5	79.2±3.5 (83.7-74.8)	70.6±2.4 (71.4-68.2)				4.40±0.37	0.24±0.08		
	9	4	78.5±3.9 (80.9-72.6)	71.5±2.8 (73.4-67.4)				3.76±0.51	0.29±0.08		
	10	5	74.3±4.0 (79.9-69.8)	67.0±3.38 (71.2-63.3)				4.1±0.45	0.17±0.06		
	11	6	66.3±6.5 (72-53.8)	57.3±6.1 (61.9-45.5)				3.24±0.68	0.31±0.11		
<b>Study II AS-3</b>											
	0	8			822±50	1.2±0.4		3.96±0.74	0.03±0.01	12.5±2.5	
	8	6	82.1±5.8 (92.3-76.4)	83.1±5.4 (92.6-78.0)	633±22	21.3±1.5		3.71±0.85	0.24±0.1	0.3±0.05	
	9	7	72.9±8 (79.9-56)	75.8±5.6 (82.5-68.6)	632±26	22.7±1.9		3.17±0.54	0.34±0.09	0.45±0.1	
	10	6	67.3±2.1 (69-64.9)	65.6±5.6 (72-60.9)	595±42	24±1.4		2.84±0.52	0.43±0.15	0.37±0.1	
<b>Study III EAS-61</b>											
	0 C	8			1541±118	0.91±0.34	100.0±0.1	4.09±0.32	0.04±0.03		
	0	8			1537±69	1.03±0.28	99.9±0.1	3.92±0.55	0.05±0.02		
	9 C	8	73.5±5.5 (80.8-66.3)	77.4±9.5 (82.1-66.4)	1092±25	31.7±4.2	70.7±9.3*	2.17±0.69	0.31±0.11		
	9	8	74.2±5.8 (80.7-66.8)	74.0±4.7 (83.1-69.4)	1048±32	34.7±1.6	65.1±6.7*	2.35±0.38	0.33±0.07		
	10	4	71.3±10.2 (81.6-58.5)	68.0±10.9 (79.6-54.5)	1055±57	31.8±3.8	64.4±4.8	2.09±0.19	0.42±0.09		
<b>Study IV OFAS3 with PIPA supplement additions</b>											
	0 C	8			1317±80	1.0±0.4	100	3.52±0.24	0.05±0.03 (0.09-.01)	8.50±1.12	
	0	8			1308±75	1.1±0.3	99.7±0.6	3.61±0.40	0.08±0.03 (0.11-0.04)	9.14±0.97	
	10 C	8	69.7±9.5 (78.5-54.8)	68.1±10.0 (79.7-49.7)	1014±665	26.6±2.5	60.8±13.3	2.12±0.49	0.59±0.30 (1.2-0.2)	0.88±0.13	
	10	8	73.0±10.1 (90.3-57.3)	77.3±12.5 (96.0-56.7)	887±34	37.7±10.1	80.6±15.0	5.00±0.65	0.35±0.11 (0.7-0.2)	9.32±1.04	
	12	4*	70.7±3.1 (74.3-66.9)	72.2±5.4 (78.4-67.1)	755±67	42.4±2.0	82.1±7.1	5.03±0.61	0.47±0.13 (0.9-0.3)	9.72±0.71	
	14	6*	69.7±9.1 (79.6-58.3)	70.0±9.4 (79.6-57.1)	790±22	47.9±1.5	77.4±9.0	4.41±0.68	0.61±0.16 (1.2-0.4)	11.61±0.95	
	16	2	72.4±3.8 (75.1-69.7)	76.7±3.5 (79.2, 74.2)	735±21	55.0 ±0		3.56±1.07	1.15±0.9 (1.8-0.5)	10.8±1.4	
<b>Study V OFAS3</b>											
	0 C	8			721 ± 19 (748-695)	3 ± 4 (13-1)	100 ± 1 (100-99)	3.59 ± 0.91 (4.46-1.67)	0.06 ± 0.03 (0.10-0.03)		
	0	8			1209 ± 76 (1307-1096)	2 ± 0 (2-1)	98 ± 4 (100-87)	3.48 ± 0.86 (4.17-1.50)	0.06 ± 0.03 (0.13-0.03)		
	6 C	8	77.8 ± 5.4 (83.5-67.3)	75.1 ± 7.7 (83.5-62.0)	547 ± 37 (594-490)	22 ± 6 (29-14)	51 ± 18 (73-24)	2.93 ± 0.80 (4.28-1.95)	0.4 ± 0.2 (0.7-0.2)		84 ± 16 (112-58)
	6	8	82.3 ± 4.6 (87.5-71.9)	83.0 ± 5.0† (87.7-72.2)	920 ± 39 (971-864)	25 ± 4 (30-21)	63 ± 17 (87-43)	3.76 ± 0.62 (4.55-2.98)	0.3 ± 0.2 (0.5-0.2)		82 ± 12 (97-67)
	9	8	73.5 ± 8.5 (83.5-61.0)	72.6 ± 10.5‡ (85.7-54.1)	900 ± 34 (962-853)	31 ± 2 (34-29)	54 ± 15 (75-25)	2.71 ± 0.69 (3.75-1.86)	0.8 ± 0.7 (2.1-0.2)		86 ± 7 (98-74)

\*C with bold letters—anaerobic control; SLR—single <sup>51</sup>Cr label; DLR—double label; <sup>51</sup>Cr and <sup>99m</sup>Tc.

Data are reported as mean ± SD (range)

\* PIPA supplements were added at weeks 7 and 11 without warming the units.

# No difference in study arms, p=0.73

@ Out of six subjects with 75% or more at 10 weeks of storage, four were tested at weeks 12 and 14; two subjects with the highest values at week 10 were tested at weeks 14 and 16.



## Clinical studies with anaerobically stored red blood cells

In the past 12 years, we have conducted five clinical studies with anaerobically stored RBC to assess the utility of anaerobic storage using an *in vivo* metric of quality of stored RBC – autologous 24-hour recovery of  $^{51}\text{Cr}$ -labelled RBC in human subjects. Studies III-V (described below) were designed as dual-arm trials in which RBC from the same subject were stored following conventional aerobic procedures (control) and under anaerobic conditions (test). We tested several experimental additive solutions as well as AS-3 (Pall Corp., Port Washington, NY, USA) in combination with the anaerobic conditions in these studies, the initial goal of which was to achieve the longest possible storage shelf-life of RBC units (Table II).

In all five studies,  $\text{SO}_2$  of Hb was reduced to below 4% by an experimental gas exchange protocol<sup>51</sup>. The gas exchange was conducted by transferring packed RBC into 1 L transfer bags, filling the bags sterily with Ar, gently agitating the bags for 10 min, and finally expressing Ar/ $\text{O}_2$ . After repeating the gas exchange six times, the RBC suspension was transferred to the original polyvinyl chloride storage bag, and placed in an anaerobic canister filled with 90% Ar /10%  $\text{H}_2$ ; a palladium catalyst was included in the canister to prevent  $\text{O}_2$  re-entry and to further deplete  $\text{O}_2$  during storage. The results of these studies are summarised below.

## Metabolic and biochemical parameters

Under conventional (aerobic) storage conditions, ATP concentration peaks at 10-20% above starting level around 3 weeks of storage and then gradually declines. Under anaerobic conditions, depending on the additive used, ATP peaks at higher levels (50-70% above the initial concentration), and this phase of ATP boost is sustained for a longer period (5-7 weeks). After the level of ATP reaches its peak, the rate of decline is similar for aerobic and anaerobic storage. Because ATP peaks later and at a higher level, however, ATP levels of anaerobic RBCs remain above the fresh level even after 6 weeks of storage.

In anaerobic storage, 2,3-DPG levels are sustained significantly longer than in conventional storage: 2,3-DPG increases to over 100% of the initial level within the first week and then declines to below the initial concentration only in week 3. The levels of haemolysis

**Table II -** Additive solutions used in clinical studies on anaerobic storage

(mM)	Study			
	I OFAS1	II AS-3	III EAS61	IV*, V OFAS3
Adenine	2	2.2	2	2
Dextrose	110	61	110	110
Mannitol	65		55	55
NaCl		70	26	26
Na citrate	20	20		
Citric acid		2		
$\text{Na}_2\text{HPO}_4$			12	12
$\text{NaH}_2\text{PO}_4$	20	20		
pH	7.2	5.8	8.3	6.5
mL added	250	200	200	200

\* In study IV, PIPA supplement was also added at weeks 7 and 11 of storage for the test (anaerobic) units. Rejuvesol (0.55 g sodium pyruvate, 1.34 g inosine, 0.034 g adenine, 0.5 g dibasic sodium phosphate monohydrate, pH 6.7-7.4; 50mL per addition, EnCyte Systems, MA, USA).

are similar for the first 6 weeks for both types of storage, but then diverge significantly beyond 8 weeks when the rate of haemolysis of aerobically stored RBC begins to increase. Phosphatidyl serine exposure increases slowly in the first 6 weeks in both aerobic and anaerobic conditions; at week 6, aerobic RBC show an exponential increase in phosphatidyl serine exposure, while for anaerobic RBC the rate of increase is more gradual and the exponential phase is delayed for about 3 weeks.

Prior to initiating study IV, we had examined the effect of pH of the experimental additive solutions on *in vitro* parameters (ATP, 2,3DPG and haemolysis) and found that good results could be obtained by acidifying an alkaline experimental additive solution (EAS61<sup>46</sup>). In study IV, in addition to using this adjusted additive with low pH (OFAS3), we added metabolic precursors (PIPA: phosphate, inosine, pyruvate, adenine; Rejuvesol, EnCyte System Inc., Braintree, MA, USA) to the storage solution to ensure an adequate supply of nutrients during a storage period of 12 weeks and beyond. In this study, we demonstrated that<sup>50</sup>:

- PIPA supplementation at 4°C (as opposed to incubation at 37°C) boosted ATP and 2,3-DPG levels within 1 week.

- ii) PIPA addition reversed the scrambling of membrane lipids by allowing the translocation of previously exposed phosphatidyl serine back to the inner leaflet of the membrane.
- iii) Addition of PIPA to the blood bag twice during anaerobic storage maintained ATP and 2,3-DPG at the level of fresh RBC and suppressed phosphatidyl serine exposure for more than 17 weeks.

## 24-hour recovery of red blood cells stored anaerobically

### Study I

Our first study utilised a hypotonic experimental additive solution (250 mL, 125 mOsm/L effective, **Table II**). In this small, pilot trial, 24-hour recovery of more than 75% was recorded for RBC stored in this additive solution anaerobically for 8 (n=6) and 9 weeks (n=4). This study provided the first evidence of the utility of anaerobic storage<sup>48</sup>.

### Study II

Our second study examined an existing additive solution, AS-3, in a serial protocol in which one unit collected from a donor was tested for 24-hour recovery three times consecutively, at weeks 8, 9 and 10 of anaerobic storage. A double volume of AS-3 additive was used to prevent the possible exhaustion of glucose under the conditions of glycolytic flux elevated for a prolonged period of time in extended storage. The results indicated that anaerobic storage is compatible with the existing additive solution for up to 9 weeks<sup>51</sup>.

### Study III

Neither additive solutions currently in use nor the experimental additives that demonstrate extended storage times<sup>43-46,142</sup> specifically aim to reduce oxidative damage. We hypothesised that anaerobic storage conditions will be synergistic with advanced additives to further prolong the shelf life of units by reducing the rate of development of storage lesions due to oxidative damage in addition to the direct effect of anaerobic conditions on the metabolic status of RBC (Figure 2). We tested this hypothesis in study III for an advanced experimental additive solution with high pH (8.4) which showed success in a 9-week storage under conventional aerobic conditions<sup>46</sup>.

To avoid the large subject-to-subject variability

inherent in studies with a small number of subjects, this study was also designed as a dual-arm trial in which RBC from the same subject were collected on two separate occasions, stored under aerobic and anaerobic conditions in a random order, and tested to isolate the effect from anaerobic storage conditions on storage duration.

Unexpectedly, our results showed that no further gain was achieved when anaerobic storage was combined with the alkaline additive solution. We also found that subjects whose RBC stored better in one arm had comparable results in the other arm of this study. Two plausible explanations for the observed lack of synergy are that: (i) the cytosol of RBCs became *too* basic for optimal function because of over-alkalinisation produced by the gas exchange (since both O<sub>2</sub> as well as CO<sub>2</sub> are removed in this process) and due to the activity of carbonic anhydrase; and (ii) factors other than oxidative damage (e.g., metabolic deficiency) limited the post-transfusion survival of the RBC<sup>51</sup>.

### Study IV

Based on *in vitro* experiments completed after study III, we conducted another dual-arm study, this time using an *acidic* experimental additive (OFAS3). In the control arm, RBC were stored with OFAS3 additive in aerobic conditions. In the test arm, RBC were stored anaerobically with OFAS3, and PIPA supplementation was added (without warming the units) at weeks 7 and 11 of storage. We measured 24-hour *in vivo* recovery of the RBC in both the test and control arms after 10 weeks of storage. Units in the test arm were re-examined after 12 weeks and, depending on the observed recovery, again at week 14 or 16 of storage (Table I)\*. For the test arm (anaerobic storage), an acceptable recovery rate was measured at week 12 for all units\*\*, and two units with good recovery at week 14 also showed adequate recovery at week 16.

\* Two were dropped from the study after 10 weeks because of lower than 75% recovery for the Test arm.

\*\* Out of 6 subjects with 75% recovery or more at 10 weeks of storage, 4 were tested at week 12 and week 14; 2 subjects with highest values at week 10 were tested at week 14 and week 16 (both subjects scored higher than 75% at week 14).

This study demonstrated that when anaerobic storage was combined with supplements of PIPA, RBC stored for 12 weeks showed acceptable survival *in vivo* and maintained high 2,3-DPG levels such that full oxygen delivery capacity would be available immediately after transfusion (and not only 8-24 hours later when transfused RBC stored conventionally recover their function *in vivo*)<sup>50</sup>.

### Study V

In this dual-arm, 8-subject study we stored leucoreduced RBC anaerobically in OFAS3 additive solution (test arm) and aerobically in AS-3 additive solution (control arm). We measured the 24-hour recovery at 6 weeks (test and control) and at 9 weeks (test only); we also determined the rate of long-term survival of transfused RBC after each of these three infusions.

The results of this study demonstrated that 6-week control and 9-week test units were equivalent in terms of 24-hour recovery, morphology scores, and ATP levels. Two subjects with relatively high haemolysis after 6 weeks (control) showed greater than 1% haemolysis after 9 weeks (test). The long-term survival rate was indistinguishable between all three infusions suggesting that for those RBC that survive the initial stress related to infusion and/or scrutiny by the reticuloendothelial system of the recipient, a normal life-span can be expected regardless of the storage methods used<sup>49</sup>.

### Summary of *in vivo* studies

The following conclusions can be drawn from the results of these five clinical studies:

- anaerobic conditions could extend the storage time by more than 50% while maintaining acceptable viability of stored RBC *in vivo*;
- anaerobic storage conditions in combination with an acidic additive solution had a synergistic beneficial effect on stored RBC; a combination with an alkaline additive conferred no additional benefit;
- ATP and 2,3-DPG could be maintained at high levels throughout the extended duration of anaerobic storage by adding PIPA supplement twice during storage without warming the units;
- maintaining high ATP levels throughout storage did not guarantee high 24-hour recovery post-transfusion;

- the level of phosphatidyl serine exposure at the time of re-infusion did not correlate with the rate of RBC survival *in vivo*;
- the significant reduction in oxidative damage combined with metabolite precursor supplements could not extend storage beyond 12 weeks for most units. Therefore, other factors – such as accumulation of toxic products or cold denaturation of RBC proteins – may be damaging RBC in storage and reducing their viability *in vivo*;
- no difference was observed in the long-term survival rates between RBC stored in aerobic and anaerobic conditions once the cells survived the first 24 hours *in vivo*.

### Effects of carbon dioxide depletion during gas exchange

The process of gas exchange used in these studies was effective in removing O<sub>2</sub> as well as dissolved carbon dioxide (CO<sub>2</sub>). Depletion of CO<sub>2</sub> may have caused alkalosis in RBC cytosol, which may have contributed to the observed increase in glycolytic flux yielding high levels of ATP<sup>51,143</sup>. To rule out this possibility, we used CO (instead of Ar) for gas exchange in order to force Hb into the R-state (COHb), while still alkalizing RBC by CO<sub>2</sub> removal. In these experiments, gas exchange with CO depleted O<sub>2</sub> and CO<sub>2</sub> (as with Ar in all other anaerobic experiments), but the ATP levels were suppressed (instead of enhanced) relative to those of the aerobic controls<sup>51</sup>.

This observation can be explained by CO binding to Hb: because the affinity of Hb to CO is 200 times higher than for O<sub>2</sub>, Hb was locked into R-state and thus released 2,3-DPG into the cytosol. This process also freed the binding domain of band 3 for PFK and other glycolytic enzymes to bind (Figure 2C). Since RBC contain neither mitochondria nor metabolic enzymes to be inhibited by CO, the reduced ATP levels can only be attributed to a reduced glycolytic flux<sup>144</sup>. On the other hand, the Hb-CO complex is extremely stable and once formed it prevents any auto-oxidation from taking place and causing oxidative damage. These results showed that even if cytosolic alkalisation caused by CO<sub>2</sub> removal played a role in elevating the level of ATP, the contribution was insignificant in comparison to the effect on glycolytic flux from the conformational transition of Hb.

### **Practical implementation of anaerobic storage**

In all clinical studies described above, the initial depletion of O<sub>2</sub> was carried out manually using an experimental protocol of gas equilibration in a large transfer bag. Deoxygenated RBC were then stored in an anaerobic canister to prevent re-oxygenation. We are now designing and fabricating a prototype blood collection/storage system to replace this manual experimental protocol. Our goal is to make this new anaerobic storage system: (i) self-contained; (ii) disposable; (iii) inexpensive; (iv) easy to gain approval under current blood banking regulations; (v) require no or minimal additional infrastructure; and (vi) require minimal departure from the current operational modality of blood banks.

The prototype system consists of a device for the initial O<sub>2</sub> depletion and a multi-layered anaerobic storage bag impermeable to O<sub>2</sub>. Initially, the system will be made compatible with packed RBC units prepared by the currently available blood collection sets. Anaerobic RBC will be prepared by sterilely connecting a unit of packed leucoreduced RBC to the O<sub>2</sub>-depletion device, passing the RBC through the device to deplete O<sub>2</sub>, and collecting the deoxygenated RBC into the anaerobic storage bag. Future versions will include a complete blood collection set incorporating the anaerobic system with additive solution, blood component bags and a leucoreduction filter. The O<sub>2</sub>-depletion device will resemble a dialysis cartridge commonly seen in renal dialysis instruments. In place of the flowing dialysate, O<sub>2</sub> sorbent granules will be packed around the hollow fibres to scavenge O<sub>2</sub> from the suspension of RBC passing inside the fibres. Deoxygenated RBC will be placed in a conventional polyvinyl chloride blood storage bag – this bag will be either laminated on both sides to make it impermeable to O<sub>2</sub>, or will be contained within an O<sub>2</sub>-barrier bag containing a sachet with an O<sub>2</sub> sorbent to compensate for possible leakage of O<sub>2</sub> and to further deplete O<sub>2</sub> during storage.

### **Utility of anaerobic storage**

The use of RBC units with a higher proportion of viable cells at the time of transfusion would benefit chronically transfused patients directly: transfusing fewer non-viable RBC reduces iron overload and may increase the clinical efficacy of transfusion.

The number of RBC units discarded due to

outdating in the developed countries is low. In the US, outdate rates are usually <2%, and most of this is due to A<sup>+</sup> and AB blood types. Furthermore, in 2007, the average age of transfused units was 19.5 days in the US nationally<sup>145</sup>, 21±11 days for patients in Intensive Care Units<sup>7</sup> and 20±11 days for trauma patients, only 18% of whom received RBC units stored for longer than 40 days<sup>146</sup>. Thus, there is little urgency to extend the shelf-life of RBC units in general transfusion therapy (with a possible exception of the military setting<sup>147</sup>). There is little doubt, however, that RBC are damaged during refrigerated storage and that this damage increases progressively with the duration of storage. There is a growing (yet controversial) concern regarding the negative consequences of transfusing "old" RBC<sup>16</sup>. Strategies to reduce the rate of development of storage lesions are particularly timely and should warrant rigorous pursuit. Extending the shelf-life of stored RBC is one way of reaching this goal. The use of RBCs with a shelf life extended by 50% under current normal practices would effectively amount to the use of relatively "fresher" blood even though the "nominal age" would be the same.

Currently, we expect anaerobic storage to benefit cardiac surgery and critical care patients as numerous recent reports have suggested an association between the age of transfused units and poor clinical outcomes in these groups of patients (for a recent comprehensive review see Lelubre *et al.*<sup>16</sup>). More general conclusions must await results of two ongoing large clinical trials of the efficacy of RBC transfusion<sup>\*\*\*</sup> aimed at determining a correlation between the age of RBC units and patients' outcomes. The potential utility of anaerobically stored RBC would not be limited to specific groups of patients and could be made universally available if an inexpensive anaerobic storage system could be developed and incorporated into the current blood banking operation without causing a major disruption to normal procedures.

### **Conclusions**

In the past, oxidative damage to RBC has been studied extensively in the context of patients suffering

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\*\*\*Red Cell Storage Duration and Outcomes in Cardiac Surgery, n=2,800; and Red Cell Storage Duration Study (RECESS), n=1,612 (<http://clinicaltrials.gov>).

from hereditary haemoglobinopathies. The mechanisms and the impact of oxidative reactions on the physiological function of RBC are now well understood. Because of its abundance and high reactivity, haemoglobin plays a central role in promoting the oxidation in RBC with oxygen (present at a relatively high concentration in the cytosol of the cell) acting as a fuel in this process. Despite multiple reports implicating or hypothesising oxidative damage mediated by denatured Hb as the major driving force in the development of storage lesions<sup>109,148</sup>, until recently, little effort has been focused on reducing oxidative damage directly. Moreover, a mere handful of reports exists unequivocally demonstrating evidence of specific oxidative damage in the early stages of RBC storage.

An attractive way to reduce oxidative damage during refrigerated storage is to eliminate for the entire duration of storage a critical substrate of oxidative reactions – oxygen. In a series of pilot clinical studies, we tested this hypothesis by stripping oxygen away from haemoglobin at the onset of storage and maintaining the anaerobic conditions throughout the storage. Our 24-hour recovery data suggest that anaerobic conditions combined with existing additive solutions can reduce the rate of development of storage lesions and extend the refrigerated storage of RBC by 50% or more. On the other hand, anaerobic storage reduces the rate of storage lesion development during storage and, therefore, has the potential to improve the functional quality of RBC stored for any given duration. In addition to these 24-hour *in vivo* recovery data, a recent comprehensive proteomic study of the early development of oxidative damage to membrane proteins and the cytoskeleton of RBC showed a significant reduction of oxidative damage in anaerobic conditions<sup>114</sup>.

At this time, there are no definitive data available to correlate the extent and/or type of oxidative damage and the viability and efficacy of stored RBC *in vivo*. It appears prudent, however, to look for strategies for combating the development of oxidative damage during storage. In pursuing this goal, storing RBC in anaerobic conditions offers an attractive alternative to the addition of anti-oxidant chemicals to the additive storage solution primarily because anaerobic storage completely avoids the safety concerns associated with infusing these chemicals into the recipient during

transfusion. A larger clinical study examining the correlations between the extent and nature of oxidative damage and the 24-hour *in vivo* recovery of stored RBC is currently underway. If a causal relationship can be established, RBC preserved in a user-friendly, self-contained anaerobic blood collection/storage system will provide more viable, "fresher" RBC to all patients receiving transfusions.

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